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# The Neuroprotective Benefits of Central Adenosine Receptor Stimulation in a Soman Nerve Agent Rat Model

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## ABSTRACT

The current regimen for treating nerve agent poisoning does not sufficiently suppress the excitotoxic activity that causes severe brain damage, especially in cases where treatment is delayed and nerve agent-induced status epilepticus develops. New therapeutic targets are required to improve survivability and minimize neuropathology after irreversible acetylcholinesterase inactivation. Earlier studies have shown that systemic delivery of adenosine agonists decreases nerve agent lethality; however, the mechanism of protection remains to be understood. The primary aim of this study was to investigate the role of central adenosine receptor (AR) stimulation in neuroprotection by directly injecting (6)-cyclopentyladenosine (CPA), an adenosine agonist specific to the A1 receptor subtype (A1R), into the brain intracerebroventricularly (ICV) in a soman seizure rat model. In addition to general A1R stimulation, we hypothesized that bilateral micro-injection of CPA into the cholinergic basal forebrain (BF) and of the adenosine A2AR agonist CGS21680 into the GABAergic ventrolateral pre-optic area (VLPO) could also suppress excitotoxic activity. The results from these studies demonstrated that centrally administered adenosine agonists are anti-seizure and neuroprotective. CPA-delivered ICV prevented seizure and convulsion in 100% of the animals. Moreover, neuropathological evaluation indicated that adenosine treatments reduced brain damage from severe to minimal. Inhibition of the BF via CPA and stimulation of the VLPO via CGS21680 had varied results. Some animals were protected by treatment; however, others displayed similar pathology to the control. Overall, these data suggest that stimulating central ARs could be an effective target for the next generation countermeasures for nerve agent intoxication.

## INTRODUCTION

Organophosphorus (OP) chemical warfare nerve agents (CWNAs), such as soman and sarin, irreversibly inhibit acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) in the cholinergic synapses and neuromuscular junctions (McDonough et al. 1997). Following exposure to CWNAs the earliest neurochemical events detectable in the central nervous system (CNS) are the inhibition of AChE and an immediate increase in brain neurotransmitter acetylcholine (ACh) levels (Shih 1982). After a longer duration of seizure activity, changes in levels of excitatory (glutamate) and inhibitory ( $\gamma$ -aminobutyric acid, GABA) amino acid transmitters are observed (el-Etri et al. 1992; Fosbraey et al. 1990; Lallement et al. 1991; O'Donnell et al. 2010; O'Donnell et al. 2011; Wade et al. 1987). Many potential inhibitory compounds and drugs along these lines of neurotransmission perturbations have been investigated (McDonough and Shih 1997; Shih 1990; Shih et al. 2003). These treatments have limited efficacy in protecting the CNS, particularly in cases of prolonged seizure activity. Therefore, investigation and exploration of new therapeutic targets for CWNA countermeasures are needed.

Adenosine is an endogenous substance that regulates multiple peripheral and central physiologic functions. It is released during normal metabolic activity into the extracellular space where it acts on adenosine receptors (ARs) (Ribeiro et al. 2002). Adenosine modulates cellular activity by stimulating specific AR subtypes that are classified according to their effect on adenylyl cyclase; A1 inhibits activity via Gai proteins and A2A enhances activity via Gas proteins (Haas et al. 2000; Sperlagh et al. 2011; St Hilaire et al. 2009). Adenosine's most notable physiologic effect occurs with the stimulation of A1Rs in the brain. Activation of central A1Rs elicits a profound inhibitory effect on neuronal excitability and synaptic transmission. Pre-synaptically, adenosine reduces the influx of calcium, which suppresses the release of glutamate. Post-synaptically, adenosine decreases neuronal excitability by inhibiting N-methyl-D-aspartate (NMDA) receptors and voltage-sensitive calcium channels (Malva et al. 2003; Ribeiro et al. 2002). A1Rs are distributed throughout numerous brain structures including the cortex and thalamus, and have the highest densities in critical cholinergic centers, such as the basal forebrain (BF), hippocampus, and striatum (Bjorness et al. 2009; Svenningsson et al. 1997). In addition to the brain, A1Rs are also widely distributed throughout the periphery. A1Rs have been detected in the heart, aorta, liver, kidney, eye, and bladder (Dixon et al. 1996). In the periphery, A1R stimulation primarily decreases heart rate and blood pressure (Schindler et al. 2005). A2ARs are also located in both the periphery and brain, but are expressed to a much lesser extent than A1Rs. In the CNS, A2ARs are primarily expressed in the basal ganglia, and less so in hippocampus, cerebral cortex, nucleus tractus solitarius, and motor nerve terminals (Schiffmann et al. 2007; Shen et al. 2009). Activation of A2ARs in the brain enhances the release of neurotransmitters and may promote GABAergic signaling (Rosin et al. 2003). Peripheral A2ARs are located primarily in the vasculature and decrease blood pressure by mediating vasodilation (Schindler et al. 2005; Tabrizchi et al. 2001).

Previous research has shown that exogenously administered adenosine provides neuroprotection from various trauma including epilepsy, hypoxia, and ischemia (Basheer et al. 2004; Cunha 2005; Lynge et al. 2000; Schubert et al. 1997; Svenningsson et al. 1997; van Helden et al. 1998; Wardas 2002). These earlier data suggest that adenosine's protective mechanism involves the partial neutralization of neuronal  $\text{Ca}^{++}$  overload that leads to cell death (Schubert et al. 1997). Adenosine's inhibitory effect on neuronal excitability has also been exploited for the treatment of drug-resistant epilepsy (Gouder et al. 2003; Huber et al. 2002; Young et al. 1994). Unfortunately, adenosine therapeutics have not been widely implemented because of the profound reductions in heart rate and blood pressure that peripheral AR stimulation triggers (Biaggioni 1992; Dunwiddie et al. 2001; Schindler et al. 2005). Despite such cardiovascular effects, van Helden et al. (1998) recognized adenosine's potential as a CWNA countermeasure. In their early study, the A1 adenosine agonist (6)-cyclopentyladenosine (CPA) was shown to reduce nerve agent lethality; intramuscular (IM) injections of CPA decreased extracellular ACh levels, diminished seizure activity, and improved survivability in a soman-induced seizure rat model. Other researchers, many of whom are affiliated with van Helden, pursued adenosine in nerve agent models, and identified its neuroprotective properties (Bueters et al. 2002; Bueters et al. 2003; Compton 2004; Joosen et al. 2004; Tuovinen 2004). However, the mechanism of protection has yet to be agreed upon. Much of the contention can be attributed to the systemic administration method. Since adenosine agonists were injected intramuscularly or subcutaneously, both central and peripheral receptors were stimulated. Consequently, marked decreases in heart rate and blood pressure accompanied the suppression of CNS hyperactivity. Some theorized that this concomitant decrease in cardiac output protected the brain since less nerve agent likely circulated to the brain (Joosen et al. 2004), while others believe that protection is actually produced by adenosine's central inhibitory effects (Filbert et al. 2005). To better understand adenosine's neuroprotective mechanism and to assess its true efficacy, it is essential that the effects of central and peripheral AR stimulation are separated. Therefore, the main goal for this project was to measure the neuroprotection offered by stimulating central ARs by microinjecting adenosine agonists directly into the brain's ventricular system.

In addition to assessing the neuroprotection that diffuse central AR stimulation offers, this project investigated if adenosinergic manipulation of particular nuclei, the BF and the ventrolateral preoptic area (VLPO) could minimize nerve agent-induced excitotoxic activity. *In vivo* models using electrical and chemical stimuli confirm that the BF is a critical center that promotes wakefulness, and its inhibition decreases brain activity (Lin et al. 2011). Furthermore, the BF excites numerous brain regions; its cholinergic neurons synapse on vital structures such as cerebral cortex, hippocampus, thalamus, amygdala, and olfactory bulb (Semba 2000). Hyperactive ACh release from BF terminals may, therefore, be a major promoter of seizure activity. Since approximately 90% of the neurons in the basalis of Meynert are cholinergic and provide the principal source of ACh to the entire cortical surface (Mesulam et al. 1983), and adenosine has been shown to suppress neuronal discharge in the cholinergic BF (Strecker et al. 2000), we hypothesize that BF A1R stimulation may attenuate seizure activity after nerve agent exposure. On the other hand, the VLPO may help suppress

nerve agent-induced excitotoxic activity. The VLPO is the brain's primary inhibitory center with GABAergic projections to the cortex and other arousal centers, such as locus coeruleus, pedunculo-pontine nucleus, lateral dorsal tegmentum, basal forebrain, periaqueductal grey, dorsal raphe, and tuberomammillary nucleus (Lin et al. 2011). The VLPO's inhibitory capacity is demonstrated by its important role in sleep promotion (Luppi et al. 2011). Studies investigating the VLPO's functional connections and sleep promoting effects have demonstrated that it can be activated by A2AR agonists such as 2-[p-(2-carbonylethyl) phenylethylamino]-5'-N-ethylcarboxamido adenosine (CGS21680) when they are administered to the subarachnoid space rostral to the basal forebrain (Basheer et al. 2004; Hong et al. 2005). We hypothesize that the inhibitory effects of VLPO stimulation via CGS21680 could minimize nerve agent-induced neuropathology.

## **MATERIALS AND METHODS**

### *Subjects/Animals*

Male Sprague-Dawley rats weighing 250 – 350 g were purchased from Charles River Labs (Kingston, NY) and were individually housed at  $21 \pm 2$  °C and  $50 \pm 10\%$  humidity with a 12-hour light – dark schedule (with lights on at 0600 h). Laboratory rodent chow and filtered tap water were freely available whenever the animals were in home cages.

### *Surgery*

Using aseptic surgical techniques, animals were prepared first with the insertion of an electronic temperature ID transponder between the shoulder blades subcutaneously (Bio Medic Data Systems Inc., Seaford, DE) and then had wire-electrodes implanted into the skull for recording brain electroencephalographic (EEG) activity. A stereotaxic frame with computer assisted guidance (Leica Microsystems Inc., Buffalo Grove, IL) was then used to drill two holes into skull and insert 26 gauge cannulae: (1) bilaterally toward the lateral ventricles (LVs) [Atlas Coordinates mm (AP, DV, L) (0.0, -4.5,  $\pm 1.5$ )], (2) bilaterally toward the BF [(-0.35, -8.5,  $\pm 2.0$ )], or (3) bilaterally toward the VLPO [(-0.5, -8.5,  $\pm 1.0$ )] (Paxinos et al. 2009) for drug administration. The rats were allowed to recover for 7 days before experimentation.

### *Soman Seizure Rat Model*

The soman-induced seizure rat model developed at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) for nerve agent-related neuroprotection studies was used for this study (Shih 1990; Shih et al. 1991). This model began with pre-treating animals with 125 mg/kg HI-6 (1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino-2-oxapropane dichloride) intraperitoneally (IP). Thirty minutes after HI-6 pretreatment, animals were challenged with a subcutaneous (SC) injection of  $1.6 \times LD_{50}$  (180  $\mu$ g/kg) soman, a dose that produces 100% seizure and convulsion. One minute later, animals were injected IM with 2 mg/kg atropine methylnitrate (AMN) and treated with microinjections of the adenosine A1 agonist CPA (LVs or BF groups) or the A2A agonist CGS21680 (VLPO group). The HI-6 and AMN injections were incorporated into this model to mitigate soman's peripheral effects and promote 24-hour survivability; seizure activity and neuropathology are not affected. CPA and CGS21680 were purchased from Tocris Bioscience (Bristol, England). Soman was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving



Ground, MD). HI-6 was purchased from Phoenix Chemical Inc. (Bromborough, England), and AMN was purchased from Wedgewood Pharmacy (Swedesboro, NJ).

The rat's brain activity was recorded from EEG and used to detect seizure onset. The electrodes implanted into the rat's skull were connected to recording leads via a connecting plug that attached to the rat's head with dental cement. At the time of experiment, the rats were placed in individual recording chambers (43 x 30 x 25 cm) where they were able to move freely. Twenty-four hours after exposure, the animals were placed again in these chambers and recorded for an additional 30 minutes. The EEG data were collected from the CDE 1902 amplifiers and analyzed using Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, England) and custom written MATLAB code. EEG data were continuously assessed by a trained technician who rated the seizure activity as absent or present.

#### Assessment of Neuropathology

Once the *in vivo* segment of the experiment was completed, the rats were anesthetized with sodium pentobarbital based euthanasia solution and perfused transcardially with saline followed by 4% paraformaldehyde in PBS. The brain was then extracted and stored in paraformaldehyde. This study implemented two different protocols for histology. The first protocol was used for the maximum tolerated dose (MTD) experiments. These brains were sectioned coronally at 50  $\mu$ m through the cannulae implantation site, Nissl stained, and then analyzed by a trained pathologist to verify the accuracy of cannulae placement and evaluate toxicity. The second protocol was used for assessing neuropathology in the subsequent soman seizure experiments. Those brains were serial sectioned at 5  $\mu$ m, stained with hematoxylin and eosin (H&E), and evaluated for neuropathology using established methodology (McDonough et al. 1995). A trained pathologist, who was unaware of treatment paradigm, analyzed and scored four brain regions, the piriform cortex, the thalamus, the dorsal and ventral hippocampus, using the standard rubric: 0 = No lesion; 1 = Minimal (1-10%); 2 = Mild (11-25%); 3 = Moderate (26-45%); 4 = Severe (>45%). To further stratify the data and obtain a more comprehensive measure of brain damage, a total score was calculated by summing the 4 regional scores. A total score of 16 indicates widespread severe damage.

#### Determination of Maximum Tolerated Doses

Since it was believed that optimal neuroprotection would be achieved at the peak of adenosine receptor stimulation, the first objective was to determine the MTD of CPA and CGS21680 that produced no toxic side-effects. The primary adverse side-effect was expected to be peripheral AR stimulation-induced cardiovascular depression. To detect such a reaction, a pulse oximeter was initially implemented for non-invasively detecting changes in heart rate and oxygenation. However, limitations intrinsic to such a device prevented the acquisition of reliable data. Therefore, alternative methods for detecting toxicity were developed. A dose level was deemed intolerable if any of the following occurred: (1) the mucus membranes, ears, eyes, nose, feet, lips, and tails became cyanotic, (2) respiration was severely depressed, (3) animal did not recover within 24 hours, or (4) death occurred.

The MTDs were determined in 3 parallel dose-response experiments, one for each injection site (LVs, BF and VLPO, respectively), as shown in Table 1. Initial doses were chosen based on data from previously published experiments that elicited neural inhibition and anticonvulsant effects with adenosine agonists (Anderson et al. 1994; Benington et al. 1995; Methippara et al. 2005; Morairty et al. 2004; Scammell et al. 2001; Thakkar et al. 2003; Yildirim et al. 2007). Four sets of rats were tested in sequential order at each injection site with 3 rats in each set. Every rat experienced two testing sessions separated by 24 hours. CPA or CGS21680 was bilaterally injected at a specified dose on day 1 at lower dose. On day 2, the animal received the same drug at an elevated dose. For group 1 testing LV's MTD, the total dose of CPA was buffered in 10 µl of multisol (48.5% H<sub>2</sub>O, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol) and administered bilaterally at a rate of 5 µl/min. For group 2 testing the BF's MTD, CPA was buffered in 2 µl of multisol and administered bilaterally at a rate of 1 µl/min. Group 3 tested the VLPO's MTD; CGS21680 was buffered in 10 µl of multisol and administered bilaterally at a rate of 5 µl/min. The volume of vehicle used for VLPO injections (10 µl) was greater than that used for the BF (2 µl) because of CGS21680's poor solubility. Multisol's safety and non-toxicity at the prescribed volumes and injection methods were verified in a separate experiment, in which three groups of 6 rats were injected with 10 µl of multisol to the LV, 2 µl of multisol to the BF or 10 µl of multisol to the VLPO. Physiologic and histological analysis determined these vehicle parameters to be safe and non-toxic.

**Table 1.** Dose parameters for determining the maximum tolerated dose (MTD) for each injection site

Group	Agonist	Animal Set 1 (N = 9)		Animal Set 2 (N = 9)		Animal Set 3 (N = 9)		Animal Set 4 (N = 9)	
1: LVs	CPA	5 µg	150 µg	290 µg	430 µg	575 µg	700 µg	850 µg	1000 µg
2: BF	CPA	1 µg	70 µg	150 µg	215 µg	290 µg	350 µg	430 µg	500 µg
3: VLPO	CGS21680	1 µg	70 µg	150 µg	215 µg	290 µg	350 µg	430 µg	500 µg

The MTDs for CPA and CGS21680 were determined in a dose escalation process for each brain target: the lateral ventricles (LVs), basal forebrain (BF) and the ventrolateral preoptic area (VLPO). There were 4 sets of animal per brain location with 3 animals per set. Each set of animals received one lower and one higher dose of an adenosine agonist separated by 24 hours. The animals were continuously monitored for signs of toxicity for 5 hours and then again at 24 hours after a second treatment. The dose escalation process stopped once toxicity was detected or the degree of physiologic response did not change.

After CPA or CGS21680 injection, brain EEG activity was recorded for 5 hours, and the behavioral response to adenosine was assessed using a modified functional observation battery (FOB) [Appendix] and a toxic sign test (Table 2). The FOB is a widely used method for assessing pharmacologic reactions; it measures mobility and the overall level of arousal/awareness (Bowen et al. 1997; Shih et al. 2006; Youssef et al. 1997). The toxic sign test detects more pathophysiologic signs such as tremors, convulsion, salivation and uncoordinated movement. Twenty-four hours after the second injection, the rat was euthanized. The optimal MTD was determined to be the minimum dose of CPA or CGS21680 that consistently produced maximum neural inhibition for each injection site.

**Table 2.** Toxic Sign Scoring System

Toxic Signs Scores	
Motor	0 = Normal 1 = Fasciculation's 2 = Tremors 3 = Convulsions
General	0 = Normal 1 = Mildly Uncoordinated 2 = Impaired Movement 3 = Prostrated
Salivation	0 = Normal 1 = Salivation
Lacrimation	0 = Normal 1 = Lacrimation
Eye	0 = Normal 1 = Nystagmus

Toxic signs were continuously scored following drug administration during the 5-hour observation period on the day of the experiment and also scored again at the 24-hour time point.

The identified MTDs were then each tested in 18 additional rats to verify that a single injection per animal produced central effects without toxicity. Three groups of 6 animals had the MTDs microinjected as follows: (1) CPA into the LV, (2) CPA into the BF, and (3) CGS21680 into the VLPO. The physiologic and behavioral responses were recorded for 5 hours after injection, the animals were then returned to standard husbandry. Twenty-four hours after microinjection, animals were deeply sedated, perfused, and histologically prepared for pathological assessment. The MTD was verified to be nontoxic, i.e., no neuronal damage beyond what is to be expected from cannulae implantation.

#### General Stimulation of A1Rs via CPA Microinjection into the Lateral Ventricles

The neuroprotection offered by widespread central AR stimulation was investigated in the soman seizure rat model after MTD determination. The treatment target sites with atlas coordinates and administration regimens are summarized in Table 3. For LVs (Table 3, row 1), 24 animals were tested in 2 groups of 12. One minute after soman exposure, group 1 received an IM injection of AMN (2 mg/kg) and an intracerebroventricular (ICV) injection of CPA (700 µg; diluted in 10 µl of multisol) at a rate of 5 µl/min. Group 2 also received AMN (2 mg/kg, IM) 1 minute after soman exposure, but was injected with multisol instead of CPA to serve as control. At 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-minute increments, behavioral responses were assessed using the FOB (Appendix), and toxic sign scores (Table 2) were recorded for a total of 5 hours after soman exposure. EEG was continuously recorded during the 5-hour observation period. Twenty-four hours later, an additional 30 minutes of EEG data were recorded for measuring final brain activity. Rats were then deeply anesthetized, euthanized by exsanguination, and histologically prepared for analysis of neuroprotection efficacy.

#### Focal Stimulation of Basal Forebrain (BF) via A1Rs

The aim for this experiment was to determine if A1R-mediated inhibition of the cholinergic BF would attenuate nerve agent-induced excitotoxicity. Testing was performed on 2 groups of 12 animals that had cannulae implanted bilaterally in the BF (Table 3, Row 2). One minute after soman exposure, both groups were injected with AMN (2 mg/kg, IM). Group 1 then received a microinjection of CPA (350 µg; diluted in 2 µl of multisol) into the BF at a rate of 1.0 µl/min. Group 2 received multisol instead of CPA to serve as control. EEG was continuously recorded. At 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-minute increments, behavioral FOB and toxic sign scores

were recorded for a total of 5 hours after soman exposure. Twenty-four hours later, following a final 30 minutes of EEG recording animals were deeply anesthetized, euthanized, perfused, and histologically prepared for analysis of neuroprotection efficacy.

**Table 3.** Description of adenosine treatment targets and administration regimens

Target Site	# of Animals	Injection location, atlas Coordinates mm (AP, DV, L)	Treatment	Total Volume	Injection Rate
LVs	2 groups of 12	(0.0, -4.5, $\pm 1.5$ )	700 $\mu$ g CPA or multisol	10 $\mu$ l	5.0 $\mu$ l/min
BF	2 groups of 12	(-0.35, -8.5, $\pm 2.0$ )	350 $\mu$ g CPA or multisol	2 $\mu$ l	1.0 $\mu$ l/min
VLPO	2 groups of 12	(-0.5, -8.5, $\pm 1.0$ )	290 $\mu$ g CGS21680 or multisol	10 $\mu$ l	5.0 $\mu$ l/min

The maximum tolerated doses of CPA for the lateral ventricles (LVs), of CPA for the basal forebrain (BF), and of CGS21680 for the VLPO were microinjected into groups of 12 rats as a treatment to prevent seizure and neuropathology 1 minute after a  $1.6 \times LD_{50}$  dose (180  $\mu$ g/kg, SC) of soman. Results for the treatment groups were compared to control groups that received multisol vehicle instead of an adenosine agonist.

#### Focal Stimulation of the Ventrolateral Preoptic Area (VLPO) via A2ARs

The goal of this experiment was to test whether or not activating the VLPO via A2AR stimulation could suppress soman-induced hyperactive neurons and reduce subsequent neuropathology. To do so, bilateral cannulae were first implanted in the VLPO 1 week prior to testing (Table 3, row 3). On the day of the experiment, treatment was prepared by diluting the A2A agonist CGS21680 (290  $\mu$ g) in 10  $\mu$ l of multisol. Two groups of 12 animals were then challenged with soman exposure followed one min later by AMN and then either CGS21680 or multisol microinjections at a rate of 5.0  $\mu$ l/min. EEG, FOB, and toxic sign data were collected for five hours as outlined for the LVs and BF groups above. Twenty-four hours after nerve agent, an additional 30 minutes of EEG data was recorded, and the rats were anesthetized, euthanized, and perfused for histology.

#### Data Analysis

Regional differences in pathology severity (i.e., normal, minimal, mild, moderate, or severe) between treatment and control groups were compared using the Chi-Square test. The total neuropathology scores (0=normal, 16=severe) for each treatment group were compared to their controls using the Mann-Whitney test, and compared between treatment groups using the Kruskal-Wallis test. Rates of seizure prevention and survival were compared using the Fisher's exact test. Differences between treatment and their controls for body temperature and latency to seizure were analyzed using the Mann-Whitney test. Statistical differences in the severity of toxic motor signs (fasciculation, tremor, convulsion) between treatment and control groups were detected using the Chi-square test. The level of significance was set at  $p < 0.05$ .

## RESULTS

### Determination of Central Maximum Tolerated Doses

The animals responded to the injections of CPA and CGS21680 with a significant reduction in central activity in a dose-dependent manner. When CPA was administered to the LVs and BF, and CGS21680 to the VLPO at the lower doses (150 µg for LVs and BF, 70 µg for VLPO), the animals became lethargic and did not move spontaneously. Doses below those values did not elicit notable reactions. As the doses increased, the animals entered a deeper state of sedation to the point where they could not be aroused for the duration of the 5-hour observation period (>430 µg for LVs, >150 µg for the BF, and between 150 - 290 µg for VLPO). Inspection of the EEG data demonstrated that their desynchronous baseline brain activity became more synchronized with lower frequencies and higher amplitudes (shown in Figure 1), similar to that of a deep sleep.

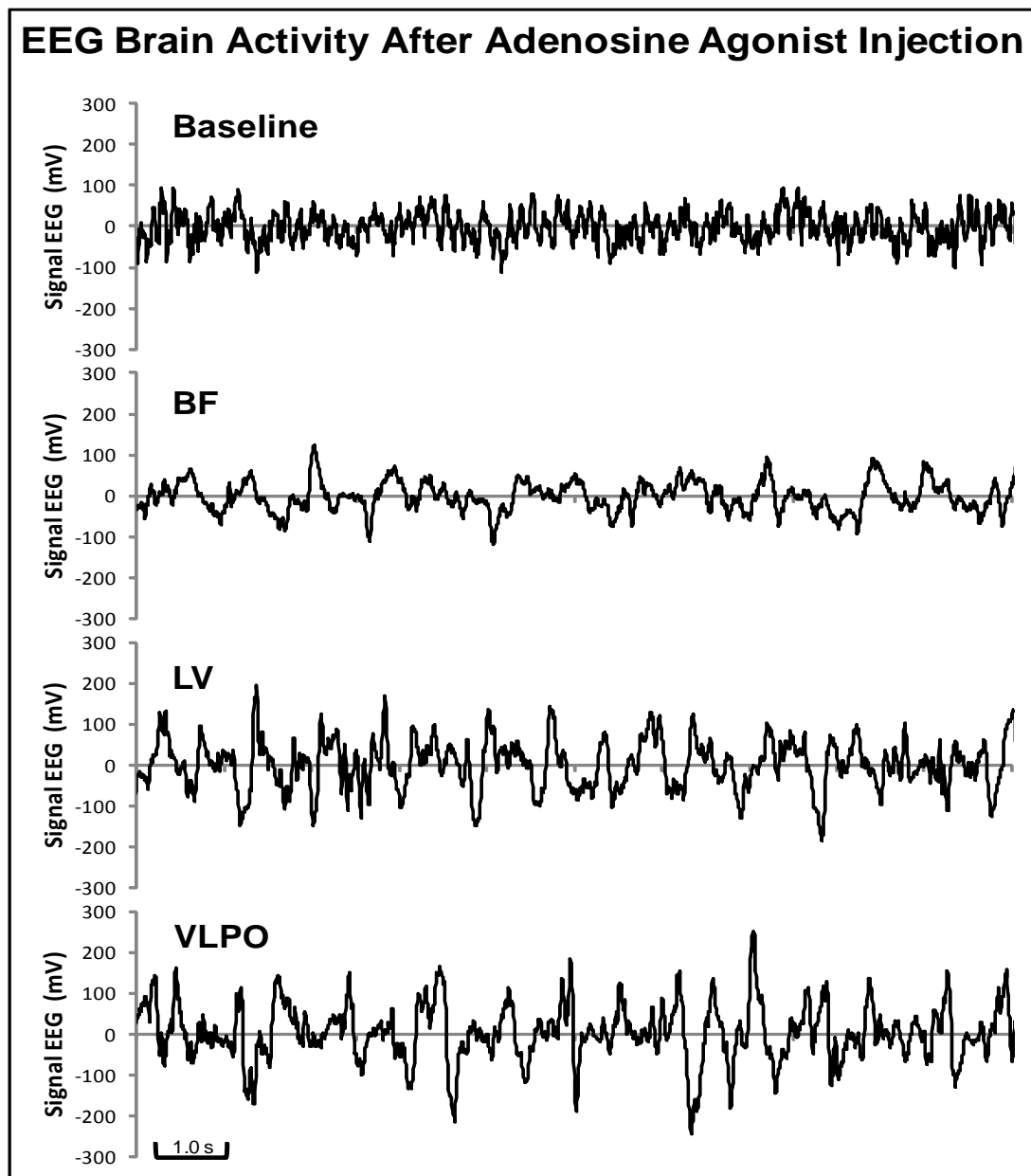
The dose-dependent behavioral response was more consistent for the LVs and BF groups compared to the VLPO group. Most of the VLPO animals responded similarly after stimulation of A2A receptors, but the latency and severity of effect were more varied. In addition, there was some residual effect from the first injection 24 hours later, particularly for the larger doses of CGS82160 into the VLPO. While the reaction to the higher dose from the second injection was not expected to be influenced by the preceding day, the severity of reaction to the adenosine agonist was slightly diminished for the second injection even though the dose was greater.

The minimum dose that produced the maximum response was determined to be the MTD for each administration level. This occurred at 700 µg CPA for the LVs group, 350 µg CPA for the BF group, and 290 µg CGS21680 for the VLPO group. At these doses, the LVs and BF groups experienced a statistically significant decrease in body temperatures during the 5-hour observation period ( $p < 0.05$ ). Whereas the animals that received only multisol decreased  $1.4 \pm 1.1$  °C on average, a likely product of normal circadian variations in temperature, the LVs group decreased  $4.7 \pm 2.2$  °C, and the BF group decreased  $3.3 \pm 0.6$  °C. The VLPO group did not have a significant decrease ( $p = 0.18$ ) in body temperature; the decrease was  $1.2 \pm 0.59$  °C.

The latency between microinjection and behavioral effects shortened as doses escalated to MTD levels. Doses at MTD into the LVs produced diminished behavioral and neural activity within 4-8 minutes, and between 8-15 minutes for the BF and VLPO MTDs. Recovery of central and motor functions varied between treatment regimens. The injection of CPA to the LVs resulted in the longest lasting suppression of neural activity; the animals could not be aroused at the final 5-hour assessment time point, but were able to recover by the next morning (>24 hours). The effects of CPA at MTD to the BF began to diminish by the end of the 5-hour period. The injection of CGS21680 at MTD to the VLPO was the shortest acting. The animals recovered cognitive and motor function within 1.5 to 2 hours after microinjection.

While the adenosine agonists were administered centrally, there appeared to be some peripheral side-effects such as a reduction in cardiovascular output and development of pallor, particularly in the LVs group. Since only peripheral and not central AR1 stimulation had been shown to have cardiovascular effects (Schindler et al. 2005), a fraction of the CPA likely escaped the CNS and entered peripheral circulation.

In contrast to the LVs group, the BF group maintained their pink skin and mucous membrane coloration. The VLPO group responded with the fewest peripheral effects: no significant decreases in respiration or changes to tissue coloration.

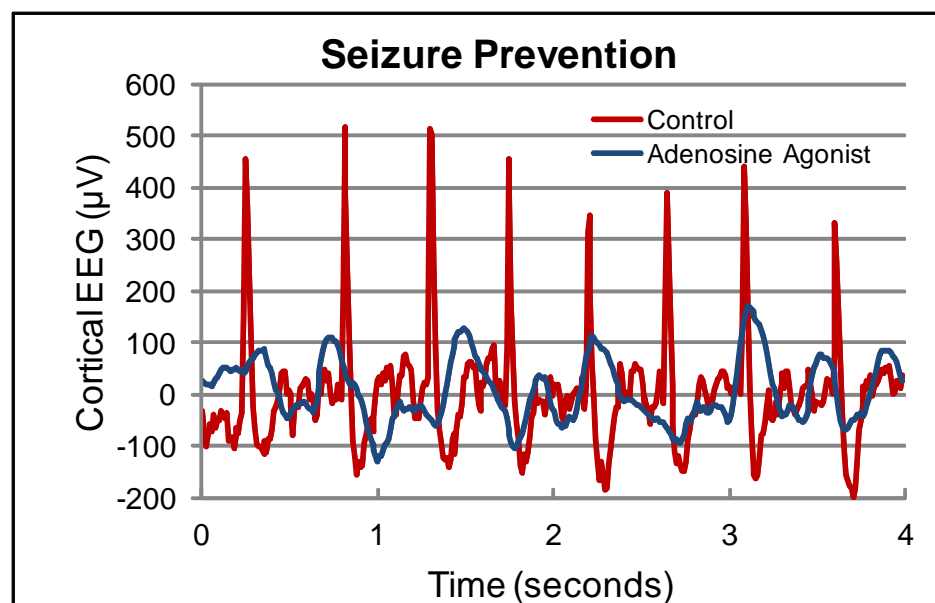


**Figure 1.** Cortical EEG tracings before adenosine injection (Baseline) and 1 hour after adenosine agonist microinjections at MTDs for 3 different locations. The A1 agonist CPA was delivered ICV to the LVs at a dose of 700  $\mu$ g or was injected directly to the BF at a dose of 350  $\mu$ g. The A2A agonist CGS21680 was directly injected into the VLPO at a dose of 290  $\mu$ g. The desynchronous EEG activity (low amplitude, high frequency) of the baseline indicates wakefulness. The adenosine agonists produced signals with higher amplitude and lower frequencies; the EEG responses are similar to deep sedation.

After transcardial perfusion and fixation, the brains were sectioned and stained with Nissl. A trained pathologist then analyzed the sections and verified that CPA or CGS21680 treatment was non-toxic and that cannula placement was grossly accurate. For most animals, cannulae were implanted accurately, and there were no signs of infection beyond what was to be expected after an aseptic surgical procedure. However, 3 LVs rats, 2 BF rats, and 1 VLPO rat developed moderate to severe infections from the surgery or had inaccurate cannulae placements. They were removed from the study.

#### Adenosine agonist to LVs after soman challenge

CPA protected the animals from soman-induced seizure and convulsions. Figure 2 shows EEG tracings from one animal that received CPA treatment and another animal that received multisol after exposure to soman. It illustrates CPA's suppression of soman-induced spike activity. As shown in Table 4A, the 12 control animals that did not receive CPA but multisol exhibited seizure onset times of  $7.2 \pm 3.1$  minutes on average after exposure to a  $1.6 \times LD_{50}$  soman challenge. All 12 control animals also developed convulsions (motor toxic sign = 3) as well as a prostrated posture (general toxic sign = 3). None of the animals that received  $700 \mu\text{g}$  CPA via ICV developed seizure. They were deeply sedated and were not responsive to any external stimuli, similar to the animals that received CPA during the MTD tests. There were no convulsions or behavioral signs of a cholinergic crisis throughout the 5-hour monitoring period. Furthermore, there were no indications of a central excitotoxicity; the EEG data resembled that of deep non-random eye movement sleep. In addition to silent neuronal activity, those treated with CPA experienced mild hypothermia. Their temperatures decreased over the 5-hour period from a baseline of  $37^\circ\text{C}$  to  $32.8 \pm 1.3^\circ\text{C}$ . That decrease in temperature after treatment was significantly different from results in the control group, which had an average temperature of  $37.2 \pm 0.8^\circ\text{C}$  after 5 hours ( $p < 0.01$ ).



**Figure 2.** The effects of CPA delivered via ICV on neuronal activity after soman exposure. Thirty minutes after HI-6 (125 mg/kg, IP) pretreatment, animals were exposed to a  $1.6 \times LD_{50}$  dose (180  $\mu\text{g/kg}$ , SC) of soman. One minute following soman exposure, the treated animal received AMN (2 mg/kg, IM) and the AR1 agonist CPA at MTD (700  $\mu\text{g}$ , ICV). The control animal received multisol instead of CPA. Whereas the control animal developed

excitotoxic brain activity (red tracing), the CPA-treated animal was protected and did not seize (blue tracing).

**Table 4.** The effects of adenosine agonists on soman-induced seizure onset time and toxic motor signs.

A. CPA or multisol vehicle injected into the lateral ventricles (LVs)

	Number Seize	Minutes to Seizure Onset Mean $\pm$ SD	Toxic Motor Sign Mean $\pm$ SD
Control	12/12	7.2 $\pm$ 3.1	3 $\pm$ 0
CPA: LVs	0/12* (p<0.01)	No Seizure Activity* (p<0.001)	0 $\pm$ 0* (p<0.001)

B. CPA or multisol vehicle injected into the basal forebrain (BF)

	Number Seize	Minutes to Seizure Onset Mean $\pm$ SD	Toxic Motor Sign Mean $\pm$ SD
Control	11/12	9.1 $\pm$ 2.0	2.9 $\pm$ 0.3
CPA: BF	6/12 (p=0.07)	20.2 $\pm$ 14.7* (p<0.01)	1.9 $\pm$ 1.2* (p<0.01)

C. CGS21680 or multisol vehicle injected into ventrolateral preoptic area (VLPO)

	Number Seize	Minutes to Seizure Onset Mean $\pm$ SD	Toxic Motor Sign Mean $\pm$ SD
Control	11/12	11.4 $\pm$ 4.9	2.9 $\pm$ 0.3
CGS21680: VLPO	8/12 (p=0.16)	40.4 $\pm$ 29.5* (p=0.01)* (p<0.01)	2.3 $\pm$ 1.0 (p=0.09)

Rats received CPA into the LVs (4A), CPA into the BF (4B), or CGS21680 into the VLPO (4C) 1 minute after exposure to a 1.6 x LD<sub>50</sub> dose (180  $\mu$ g/kg, SC) of soman. Latency to seizure was recorded and toxic motor signs scored. A toxic motor sign of 0 indicates normal behavior, whereas a score of 3 designates convulsions (see Table 2). Statistically significant differences between treatment and control groups are indicated by \* (P<0.05).

CPA's protection from soman-induced seizure and convulsion continued over-night. Table 5A displays survival and neuropathology data for the LVs group. Ten of the 12 animals that received CPA survived to the 24-hour time point and did not show signs of toxicity. Eight of the 10 treated animals were awake and aware of their surroundings; the other 2 were still in a sleep-like state at the 24-hour time point. Only 1 of the 12 control animals survived 24 hours and was mildly uncoordinated (general toxic sign = 1) at that time. Applying the Fisher's exact test, the number of surviving animals in the treated group was significantly higher than in the control group (p<0.01). The neuropathology for that control animal indicated moderate to severe damage in the four assessed areas for a total score of 13 (0 = normal, 16 = most severe damage). The CPA-treated animals on average had a pathology score of 4.9 $\pm$ 1.5 [N=10]. Although the substantial reduction in brain pathology was not significantly different from the control group (due to control N=1), the LVs treatment pathology scores were significantly (p<0.01) better than the BF controls (Table 5B) and the VLPO controls (Table 5C).



**Table 5.** The effects of adenosine agonists on soman-induced lethality and neuropathology scores at twenty-four hours.

A. CPA injected into the lateral ventricles (LVs)

	24 Hour Survival	Neuropathology - Group Mean $\pm$ SD				
		Piriform	Thalamus	Dorsal Hippocampus	Ventral Hippocampus	Total
Control	1/12	4	3	3	3	13
CPA: LVs	10/12* ( $p < 0.01$ )	2 $\pm$ 0.7	0.5 $\pm$ 0.5	1.2 $\pm$ 0.8	1.2 $\pm$ 0.6	4.9 $\pm$ 1.5 ( $p = 0.1$ )

B. CPA injected into the basal forebrain (BF)

	24-Hour Survival	Neuropathology - Group Mean $\pm$ SD				
		Piriform	Thalamus	Dorsal Hippocampus	Ventral Hippocampus	Total
Control	9/12	3.57 $\pm$ 1.1	2.86 $\pm$ 1.3	3.14 $\pm$ 1.2	3.57 $\pm$ 1.1	13.1 $\pm$ 4.6
CPA: BF	6/12 ( $p = 0.4$ )	1.7 $\pm$ 1.9	1.3 $\pm$ 1.5	1.7 $\pm$ 1.9	1.7 $\pm$ 1.9	6.3 $\pm$ 7.0 ( $p = 0.13$ )

C. CGS21680 injected into ventrolateral preoptic area (VLPO)

	24-Hour Survival	Neuropathology - Group Mean $\pm$ SD				
		Piriform	Thalamus	Dorsal Hippocampus	Ventral Hippocampus	Total
Control	7/12	3.7 $\pm$ 0.8	3.0 $\pm$ 1.0	3.57 $\pm$ 1.1	3.57 $\pm$ 1.1	13.9 $\pm$ 3.9
CGS21680: VLPO	7/12 ( $p = 1$ )	2.0 $\pm$ 2.0	1.1 $\pm$ 1.7	2.0 $\pm$ 2.0	2.0 $\pm$ 2.0	7.1 $\pm$ 7.3 ( $p = 0.07$ )

Twenty-four hours after soman exposure and adenosine treatment, animals that survived were perfused and prepared for histology. A trained neuropathologist graded 4 of the brain regions for damage: the piriform cortex, thalamus, dorsal and ventral hippocampus. A score of 0 indicates no damage, 4 indicates severe damage in each brain area. Total pathology scores for the treatment group were not significantly different from the control according to the Mann-Whitney test ( $p > 0.05$ ).

\* Indicates statistically different responses between treatment and control groups.

Adenosine agonist CPA to the BF after soman challenge

The animals that received CPA to the BF as treatment for soman exposure experienced variable protection; seizure and motor response results are reported in Table 4B. Unlike the LV group that had 100% seizure prevention, 6 of the 12 animals (50%) in the BF treatment group were protected from seizure. According to the Fisher's exact test, that rate of seizure prevention is not statistically significant ( $p = 0.07$ ). Those protected animals displayed sleep like behavior similar to the LV group. Although 6 treated animals did go into seizure, their latency to seizure onset (20.2 $\pm$ 14.7 minutes) was delayed compared to the control group (9.1 $\pm$ 2.0 minutes). When all animals from the treated and control groups are compared, the difference in seizure latency is statistically significant ( $p < 0.01$ ). One of the 12 control animals did not develop EEG

seizure activity but did display signs of soman poisoning: impaired movement, tremors and convulsions. Three of the 6 CPA-treated animals that did not seize produced no peripheral signs of toxicity. The other 3 treatment animals did eventually develop mild tremors approximately 30 - 45 minutes after soman exposure. The difference in body temperatures between the CPA-treated ( $34.3 \pm 2.3$  °C [N=12]) and control BF ( $36.4 \pm 1.0$  °C [N=12]) groups was statistically significant ( $p < 0.05$ ).

The 24-hour survival and neuropathology results for the BF group are described in Table 5B. Six of the 12 animals treated with CPA survived to the 24-hour time point, 4 of which did not seize the day before. The BF multisol control group experienced better survivability than the LVs; nine of the 12 BF control animals survived to the next day. While the BF-treated group experienced greater lethality than the LV-treated group (6/12 vs. 2/12), the localized stimulation of BF ARs provided neuroprotection in addition to preventing seizure. Treated animals that did not seize had zero to minimal neuropathology; their total pathology scores were 0, 0, 4, and 4. Similar to what was observed in the control animals that seized, those treated with CPA and seized also developed severe brain damage; their total pathology scores were 14 and 16. Overall, the pathology scores between the control and treated groups were not statistically significant ( $p = 0.11$ ).

#### Adenosine agonist CGS21680 to the VLPO after soman challenge

Similar to the BF-CPA treatment group, the VLPO group treated with the A2A agonist CGS21680 displayed mixed efficacy. As shown in Table 4C, complete anti-seizure protection was provided for 4 of the 12 treated animals. Even though seizure was not entirely prevented for the other 8 treated animals, those receiving CGS21680 treatment exhibited delayed onset to seizure for an average of  $40.4 \pm 29.5$  minutes, significantly longer than the control group's  $11.4 \pm 4.9$  minute average. One of the control animals did not go into seizure as indicated by EEG; however, that animal did develop behavioral signs of toxicity, including impaired movement and convulsions. Four of the 8 treated animals that did seize had substantially longer latencies; they averaged 66 minutes. Since that latency to seizure roughly corresponds to the duration of the drug's effect in non-exposed animals (MTD study), CGS21680's pharmacokinetics may be the primary factor that determines neuroprotective efficacy. While successful in some animals, CGS21680 appeared to have little to no effect on 2 animals; they developed seizure at 11 and 15 minutes after exposure. The hypothermic response was minimized in the VLPO treatment group. Those treated with CGS21680 had an average body temperature of  $36.2 \pm 1.0$  °C, not significantly different from the control group's average of  $36.4 \pm 1.3$  °C.

The VLPO group's 24-hour survival and neuropathology are shown in Table 5C. The CGS21680-treated animals had the same survival rate as the control group; seven of the 12 animals survived 24 hours and were prepared for histology. Four of the 7 surviving treated animals did not seize the day before, nor did they develop signs of significant neuropathology; their total pathology scores were 0, 0, 0, and 7. The 3 other treated animals that seized the day before and survived developed moderate to severe brain damage; their total pathology scores were 12, 15, and 16. The control group's neuropathology was similar to the LVs and BF controls; those 7 animals had an average

total pathology score of  $13.9 \pm 3.9$ . The total pathology scores for all 7 surviving treated animals were not statistically significant from the control group ( $p = 0.10$ ).

## DISCUSSION

This project investigated new neural pathways for protecting the brain from seizure and neuropathology after nerve agent poisoning. We hypothesized that excitotoxic brain activity could be suppressed by 1) widespread stimulation of A1Rs with CPA delivered ICV, 2) focal stimulation of BF A1Rs with direct CPA microinjections, or 3) focal stimulation of VLPO A2ARs with direct CGS21680 microinjections. We believed the greatest potential for neuroprotection would be achieved when the maximum numbers of ARs were stimulated. Therefore, the first objective was to determine the MTDs for each treatment regimen. After determining the MTDs, both general and focal AR stimulations were tested in a soman seizure rat model with success; seizures and neuropathology were reduced with adenosine agonist treatment. Although direct brain injections of a treatment are not feasible outside the laboratory, this work demonstrated that central AR stimulation is a promising new therapeutic mechanism for countering nerve agent neuropathology.

General stimulation of central ARs with CPA microinjected ICV provided the most consistent protection against a soman challenge. The prevention of a cholinergic crisis can most likely be attributed to adenosine's pre- and post-synaptic actions. A1R pre-synaptic stimulation inhibits the release of ACh and glutamate, thereby limiting the accumulation of excitatory neurotransmitters in the synaptic cleft. A1R post-synaptic stimulation further suppresses neuronal activity and decreases  $\text{Ca}^{++}$  influx. Both of these actions may block nerve agent-induced hyper-excitatory activity that typically leads to neuropathology. This link between seizure prevention and neuroprotection is supported by the significant reduction in total neuropathology scores from the severe to minimal.

Adenosine agonist treatment to the BF and VLPO displayed mixed results. While some treated animals were completely protected from seizure or convulsion, others had the same reaction to soman that the control animals did. These variable results are consistent with the data collected from the dose escalation procedure for determining the MTD. While some animals were profoundly affected by a certain dose, others in that same group may have had very little reaction. The most probable explanation for this variation is cannulae placement inconsistencies. The BF and VLPO are very small targets to hit accurately ( $\sim 0.5\text{mm}^3$ ), even with a computer-assisted stereotaxic device/procedure. Differences between animal shape, size and skull features exacerbate the likelihood for placement of the cannulae outside the effective region. Deviation in expected responses during the MTD tests may also be due to the desensitization of AR stimulation after the first day of testing. Because of this effect, future dose-determination studies should extend recovery times or only inject once per animal.

In addition to implantation accuracy, other factors likely contributed to the variable efficacy. Results from the MTD studies demonstrated that the effects of CGS21680 on the VLPO wear off approximately 1.5 - 2 hours after injection, much less than what was observed for CPA via ICV (duration 5+ hours). The VLPO's diminishing GABAergic

activity is a likely factor for the generation of seizure in some animals. Once the CGS21680 was cleared, the VLPO entered an inactive state and its widespread inhibitory projections were silenced. To better protect the animal from seizure for longer periods of time, future treatment protocols should investigate a regimen with multiple dosing schedules or test alternative agonists with greater and longer lasting affinity to VLPO A2A receptors.

Previous research investigating the therapeutic benefits of adenosine agonists was confounded by the negative peripheral side-effects such as bradycardia and hypotension (Bueters et al. 2003; van Helden et al. 1998). Those authors concluded that adenosine's neuroprotective benefit was likely caused by the concomitant decline in cardiac output which decreased the amount of nerve agent that circulated to the brain. To establish that neuroprotection is achieved by the stimulation of central A1Rs, we directly injected adenosine agonists into the brain. While we cannot make any conclusive statements regarding centrally administered adenosine agonists' effect on cardiac output, it is certain that nerve agent was circulated in the brain; histology did not indicate ischemia, and the mucus membranes and tissue were perfused with blood. It is more likely that adenosine's anti-seizure efficacy is provided by pre- and post-synaptic effects rather than by cardiovascular factors.

One observable side-effect of CPA treatment is the steady decrease in body temperature from 37 °C to approximately 28-30 °C over a period of 5 hours. The effect that this decrease in body temperature has on seizure and pathology prevention is unknown. However, hypothermic conditions are known to affect cellular activity (Geeraerts et al. 2009). In some instances, the change in cellular activity can be beneficial, particularly for cardiac trauma and brain injury (McIntyre et al. 2003; Peterson et al. 2008; Schwartz et al. 2012). Furthermore, decreasing body temperature has been shown to suppress seizure activity (D'Ambrosio et al. 2013; Liu et al. 1993). Consequently, adenosine's seizure prevention capacity may not be fully attributable to a decrease in neurotransmitter release or post-synaptic inhibition. It is, therefore, necessary to conduct additional studies where body temperature is a controlled variable so that the neuroprotective mechanism can be better understood.

The experiments conducted for this study demonstrated the neuroprotective benefits of stimulating brain ARs in a soman-induced seizure rat model. However, direct brain injections of adenosine agonists are not clinically feasible; drugs need to be administered intramuscularly in the field. To begin translating this therapy, future research needs to investigate alternative administration and dosing protocols. Experiments should test if the same neuroprotective benefits can be elicited when CPA is injected systemically. To prevent negative peripheral side effects while maintaining the positive central effects, an adenosine antagonist that is impermeable to the blood brain barrier could be co-administered. Furthermore, to enhance survivability, it is recommended that greater doses of AMN be administered to further suppress the nerve agent's peripheral effects. Beyond investigating alternative administration methods, additional research is needed to obtain a deeper understanding of the neuroprotective mechanisms. Although there are unresolved questions, these encouraging findings motivate further research into this novel therapeutic target.

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